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ScienceDirect
Developmental Biology 315 (2008) 355–368
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

The *Arabidopsis* SDG4 contributes to the regulation of pollen tube growth by methylation of histone H3 lysines 4 and 36 in mature pollen

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Received for publication 4 July 2007; revised 14 December 2007; accepted 17 December 2007
Available online 5 February 2008

Abstract

Plant SET domain proteins are known to be involved in the epigenetic control of gene expression during plant development. Here, we report that the *Arabidopsis* SET domain protein, SDG4, contributes to the epigenetic regulation of pollen tube growth, thus affecting fertilization. Using an SDG4–GFP fusion construct, the chromosomal localization of SDG4 was established in tobacco BY-2 cells. In *Arabidopsis*, *sdg4* knockout showed reproductive defects. Tissue-specific expression analyses indicated that *SDG4* is the major ASH1-related gene expressed in the pollen. Immunological analyses demonstrated that SDG4 was involved in the methylation of histone H3 in the inflorescence and pollen grains. The significant reduction in the amount of methylated histone H3 K4 and K36 in *sdg4* pollen vegetative nuclei resulted in suppression of pollen tube growth. Our results indicate that SDG4 is capable of modulating the expression of genes that function in the growth of pollen tube by methylation of specific lysine residues of the histone H3 in the vegetative nuclei.

Keywords: *Arabidopsis*; SDG4; Histone methylation; Pollen tube growth; SET domain

Introduction

During plant development, the epigenetic regulation of gene expression is influenced by the state of the chromatin structure (reviewed in Goodrich and Tweedie, 2002; Reyes et al., 2002). Chromatin remodeling can alter the DNA-histone interaction via association between transcription factors and specialized multi-protein complexes. Chromatin modifying complexes contain characteristic amino acid motifs such as SET, PHD-finger, bromodomain and chromodomain, among others. The SET domain, first identified in the *Drosophila* SU(VAR)3-9, the Pc-G protein E(Z) and the trx-G protein TRITHORAX, is a histone methyltransferase domain responsible for the specific methylation of lysine residues on histone H3 and histone H4. To date, several SET domain methyltransferases have been identified from yeast to human and can be categorized into either an activator or a repressor. Generally, H3K4 and H3K36 methyltransferases act as activators, whereas H3K9, H3K27 and H4K20 methyltransferases are known to function as repressors (Fuchs et al., 2006; Jenuwein, 2006).

A classification of SET domain proteins in plants was proposed based on protein domain organization and phylogeny (Springer et al., 2003). *Arabidopsis* SET domain proteins, on the other hand, were previously assigned to four classes, namely E(Z) (enhancer of zeste), SU(VAR)3-9, TRX (trithorax) and

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.12.016

ASH1 (absent, small or homeotic 1), based on the homology of their SET domains (Baumbusch et al., 2001). Histone methyltransferase activity in *Arabidopsis* is best exemplified by KRYPTONITE (KYP) (Jackson et al., 2002), ATX-1 (Alvarez-Vanegas et al., 2003), SDG8 (Zhao et al., 2005) and the PcG proteins MEDEA, CURLY LEAF and SWINGER (Grossniklaus et al., 1998; Köhler et al., 2003; Makaverich et al., 2006). Recent studies reported that the three E(Z) homologues CURLY LEAF, MEDEA and SWINGER belong to different Polycomb group complexes but are capable of regulating common target genes in different stages of plant development (Makaverich et al., 2006). CURLY LEAF was previously shown to play a role in the stable repression of the floral homeotic gene *AGAMOUS* (Goodrich et al., 1997). Similarly, VERNALIZATION 2 and EMBRYONIC FLOWER 2 are Polycomb group proteins that repress flower development in *Arabidopsis* (Gendall et al., 2001;Yoshida et al., 2001; Moon et al., 2003; Bastow et al., 2004; Chanvivattana et al., 2004). Polycomb group proteins such as fertilization-independent endosperm or fertilization-independent seed have also been shown to function in endosperm development, seed coat formation, fruit elongation and even partial embryo development in the absence of fertilization (Luo et al., 1999; Ohad et al., 1999; Guitton et al., 2004; Katz et al., 2004).

In the SU(VAR)3-9 class, KRYPTONITE was reported to possess histone methyltransferase activities (Jackson et al., 2002) and is necessary for the maintenance of DNA methylation but not its establishment (Malagnac et al., 2002). ATX-1, a homolog of *Drosophila* TRX, is a regulator of *Arabidopsis* floral organ development and has histone H3K4 methyltransferase activity (Alvarez-Vanegas et al., 2003). An ASH1 homologue, SDG8, was shown to contribute in the control of flowering time by regulating *FLOWERING LOCUS C* expression through specific methylation of histone H3 K36 (Zhao et al., 2005).

Majority of the *Arabidopsis* SET domain proteins previously characterized were shown to participate in the floral development pathway (Goodrich et al., 1997; Gendall et al., 2001; Yoshida et al., 2001; Malagnac et al., 2002; Jackson et al., 2002; Alvarez-Vanegas et al., 2003; Moon et al., 2003; Bastow et al., 2004; Chanvivattana et al., 2004; Katz et al., 2004; Zhao et al., 2005). Baumbusch et al. (2001) performed a general expression analysis of the *AtSET* genes that they identified. All 20 active genes tested were shown to be expressed in floral buds, and most of them were found in flowers and seeds. Important developmental pathways take place in the floral meristem, which later differentiates into floral organs. The male reproductive organ, the stamen, obviously has significant functions in the life cycle of plants. Mutations in the genes that control proper stamen development can result to conversion of the stamens to another type of organ, such as those depicted in the ABC model of flower development (Coen and Meyerowitz, 1991). Furthermore, mutations in genes that are involved in anther and pollen development, such as *MS1* (Wilson et al., 2001; Ito and Shinozaki, 2002) and *MMD1*/*DUET* (Reddy et al., 2003; Yang et al., 2003) can cause flower sterility and thereby a failure to reproduce sexually.

We describe here the characterization, expression and functional analyses of an *Arabidopsis* SET domain protein, SDG4 (At4g30860). Our interest on SDG4 originated from our initial search for proteins containing specific domains associated with the chromosome, which may participate in chromatin remodeling or modifying mechanisms. The occurrence of a PHD-finger and a SET domain in SDG4 made it a suitable candidate for studying chromatin factors involved in *Arabidopsis* development. In the present paper, we show that SDG4 is a chromatin-associated protein that functions in the maintenance of methylated histone H3 K4 and K36 levels in the mature pollen grain. Regulation of gene expression in the vegetative nucleus of the pollen grain is crucial since the vegetative nucleus contain genes that are responsible for pollen germination and growth of the pollen tube, which in turn assures delivery and release of the sperm cells into the embryo sac for fertilization. In most of the pollen grains observed in *sdg4*, pollen tube lengths were reduced resulting to unfertilized ovules. Our results point to the possible function of SDG4 in regulating genes in the vegetative nucleus by methylation of lysines 4 and 36 of the histone H3 in the mature pollen.

Materials and methods

Cloning of SDG4 cDNA

A full-length cDNA for *SDG4* (RAFL07-08-N08), obtained from RIKEN BioResource Center (Seki et al., 2002), was cloned using the Gateway cloning technology (Invitrogen). SDG4–GFP expression clones were constructed using pGWB5 (for C-terminal fusion) or pGWB6 (for N-terminal fusion) binary vectors containing a GFP expression cassette under the control of a 35S promoter and *NOS* terminator.

Generation of transgenic tobacco BY-2 cell lines

Tobacco Bright Yellow-2 (BY-2) cells were cultured in modified Linsmaier and Skoog (LS) medium in a rotary shaker at 25 °C in the dark. The SDG4 expression clone was electroporated into *Agrobacterium tumefaciens* EHA101 and was introduced into tobacco BY-2 cells via the *Agrobacterium*-mediated transformation. An overnight *Agrobacterium* suspension culture was mixed with 3-day-old tobacco BY-2 cells and incubated for 3 days at 25 °C. Stable transformants were selected using solid LS medium containing 500 mg/ml carbenicillin and 7.5 mg/ml hygromycin.

Localization and dynamic analysis

Stably transformed tobacco BY-2 cells were used for time-lapse observation of SDG4 localization and dynamics. Actively growing cells of about 2 to 3 days old were suspended in fresh LS medium placed in a coverglass-bottom dish (Matsunami). Observation was performed at a 3-min time interval using an inverted fluorescence microscope (Olympus IX-71), with image acquisition controlled by the software Metamorph ver.6.2 (Universal Imaging Corp.).

Plant materials, growth conditions and T-DNA analysis

All *Arabidopsis* plants used were derived from the Columbia ecotype (Col-0). A T-DNA insertion line (SALK 128444) was obtained from the *Arabidopsis* Biological Resource Center (Alonso et al., 2003). Seeds were germinated in Murashige and Skoog (MS) medium (MS salts, 1% sucrose, 0.8% agar, 0.05% MES-KOH pH 5.7 and B5 vitamins: 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 10 mg thiamine–HCl and 100 mg *myo*-inositol). Total genomic DNA from each plant was isolated from young leaves and was used as template for PCR analysis.

The presence of the insertion in the *SDG4* gene was confirmed using *SDG4* gene-specific primers flanking the predicted insertion (Fig. 4A), in combination with a T-DNA left border (LBa1) primer.

RT-PCR

Total RNA was extracted from different *Arabidopsis* organs such as roots, leaves, stems, mature flowers and floral buds using the RNeasy Plant Mini Kit (Qiagen). mRNA was isolated using the PolyATtract mRNA Isolation Systems (Promega) and was utilized as template for cDNA amplification using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). *SDG4* expression was detected with primer set F 5′-TGTCTTGTTTGCCATAAGCCG-3′ and R 5′-TGTGTCTAGCC GCCAATCAGT-3′, which generates a 303-bp cDNA fragment. The *ACTIN* gene was amplified using the primers 5′-AGAGATTCA-GATGCCCAGAAGTC TTGTTCC-3′ and 5′-AACGATTCCTGGACCTGC-CTCATCATACTC-3′. PCR was performed using Takara Ex Taq polymerase for 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min.

In situ hybridization

The template for *in vitro* transcription of an *SDG4* antisense probe was derived from a 312-bp fragment corresponding to the last exon and 3′UTR of the

A

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  1 MLDLGNMSMSASVALTCCPSFLPAASGPELAKSINSPENLAGECNGKHLP
 51 MIPPEEEVKDIKIANGVTAFTRKQNPSDRVKKGFVLDDHVKDWVKRRVAS
101 GVSESTCFLPFLVGAKKMVDCLVCHKPVYPGEDLSCSVRGCQGAYHSLCA
151 KESLGFSKSSKFKCPQHECFVCKQRTQWRCVKCPMAAHDKHSPWSKEILH
201 LKDQPGRAVCWRHPTDWRLDTKHAVAQSEIEEVFCQLPLPYVEEEFKIDL
251 AWKDSVVKEDPPSYVHIRRNIYLVKKKRDNANDGVGCTNCGPNCDRSCVC
301 RVQCISCSKGCSCPESCGNRPFRKEKKIKIVKTEHCGWGVEAAESINKED
351 FIVEYIGEVISDAQCEQRLWDMKHKGMKDFYMCEIQKDFTIDATFKGNAS
401 RFLNHSCNPNCVLEKWQVEGETRVGVFAARQIEAGEPLTYDYRFVQFGPE
451 VKCNCGSENCQGYLGTKRKEPNCLVVSWGAKRRRLFHRPIARKPQQD
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B

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hASH1          KIRSNVYVD-VKP-LSGYEATTCNCKKPDDDTRKGCVDDCLNRMIFAECSPNTCPCGEQC
mouseASH1      KIRSNVYVD-VKP-LSGYEATTCNCKKPDDDTRKGCGDDCLNRMIFAECSPNTCPCGEQC
DroASH1        KIRTNVYAESVRPNLAGFDHPTCNCKNQGE---KSCLDNCLNRMVYTECSPSNCPAGEKC
hTRX5          HIKVNKPYGKVQIYTADISEIPKCNCKPTDENPCGFDSECLNRMLMFECHPQVCPAGEFC
SDG4           HIRRNIYLVKKKRDNANDGVGCTNCG-------PNCDRSCVCRVQCISCSKG-CSCPESC
                :*: *      :   :.                .   .*: *:   .*    *.. * *

hASH1          CNQRIQRHEWVQCLERFRAEEKGWGIRTKEPLKAGQFIIEYLGEVVSEQEFRNRMIEQYH
mouseASH1      CNQRIQRHEWVQCLERFRAEEKGWGIRTKEPLKAGQFIIEYLGEVVSEQEFRNRMIEQYH
DroASH1        RNQKIQRHAVAPGVERFMTADKGWGVRTKLPIAKGTYILEYVGEVVTEKEFKQRMASIYL
hTRX5          QNQCFTKR-QYPETKIIKTDGKGWGLVAKRDIRKGEFVNEYVGELIDEEECMARIKHAHE
SDG4           GNRPFRKEKKIKIVK---TEHCGWGVEAAESINKEDFIVEYIGEVISDAQCEQRLWDMKH
                *: : :.      :   :   ***: :   :    :: **:**:: : :   *:

hASH1          N-HSDHYCLNLDSGMVIDSYRMGNEARFINHSCDPNCEMQKWSVNGVYRIGLYALKDMPA
mouseASH1      N-HSDHYCLNLDSGMVIDSYRMGNEARFINHSCDPNCEMQKWSVNGVYRIGLYALKDMPA
DroASH1        N-DTHHYCLHLDGGLVIDGQRMGSDCRFVNHSCEPNCEMQKWSVNGLSRMVLFAKRAIEE
hTRX5          NDITHFYMLTIDKDRIIDAGPKGNYSRFMNHSCQPNCETLKWTVNGDTRVGLFAVCDIPA
SDG4           KGMKDFYMCEIQKDFTIDATFKGNASRFLNHSCNPNCVLEKWQVEGETRVGVFAARQIEA
                :  ...*   :: .  **.   *. .**:****:***   ** *:*  *: ::*   :

hASH1          GTELTYDYNFHSFNVEKQQLCKCGFEKCRGIIGGKSQRVNGLTSSKNSQPMATHKKSGRS
mouseASH1      GTELTYDYNFHSFNVEKQQLCKCGFEKCRGIIGGKSQRMNGLPSHKGSQSSSTHRKSARA
DroASH1        GEELTYDYNFSLFNPSEGQPCRCNTPQCRGVIGGKSQRVKPLP-------AVEAKPSGEG
hTRX5          GTELTFNYNLDCLG-NEKTVCRCGASNCSGFLGDRPKTSTTLS---------SEEKGKKT
SDG4           GEPLTYDYRFVQFGPEVK--CNCGSENCQGYLG-------------------TKRKEPNC
                *  **::*.:  :. .    *.*.  :* * :*                    .   .
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C

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HAT3.1         IFCAKCGSKDLSVDND-IIL-CDG-FCDRGFHQYCLEPPLRKEDIPP-DDEGWLCPGCDK
ZmHOX2a        IFCAICGSKDVTSQND-IIL-CDG-ACDRGFHQNCLSPPLLTEEIPP-GDEGWLCPACVC
MS1            IECE-CG---ATEEDGERMVCCD--ICEVWQHTRCVG-VQHNEEVPR----IFLCQSCDQ
SDG4           VDCLVCHKPVYPGED----LSCSVRGCQGAYHSLCAKESLGFSKSSKFKCPQHECFVCKQ
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Fig. 1. Primary structure and domain organization of the SDG4 protein. (A) SDG4 amino acid sequence showing the position of protein domains: PHD (red), SET (blue), AWS (orange) and Post-SET (brown). (B) Alignment of the SDG4 SET domain with that of other SET proteins. The conserved GWG motif and cysteine-rich regions are highlighted in blue and red, respectively. Asterisks (*) indicate positions of highly conserved residues; colons (:) and periods (.) denote conservation of strong and weaker amino acid groups, respectively. hASH1 (GenBank accession AAF68983), *Drosophila* (GenBank accession AAF49140), mouse (GenBank accession NP619620) and human trithorax (GenBank accession CAB45386). (C) PHD domain alignment. The PHD consensus sequences from PHD-containing transcription factors including SDG4 are represented by blue and red characters. HAT3.1 (GenBank accession CAA49263), ZmHOX2a (GenBank accession CAA61909) and MS1 (GenBank accession CAC69663).

SDG4 gene. This fragment was inserted into a TOPO-TA cloning vector pCR2.1-TOPO (Invitrogen) containing a T7 promoter sequence. A digoxigenin-labeled antisense probe for *SDG4* was synthesized with Ampliscribe T7 Flash Transcription Kit according to manufacturer's instruction (Epicentre). The RNA probe was hydrolyzed by alkali treatment to produce fragments less than 400 bp.

In situ hybridization with *Arabidopsis* floral tissues was performed using a modified protocol adapted from the Meyerowitz laboratory (described by Weigel and Glazebrook, 2002). *Arabidopsis* inflorescences including various stages of flower buds were fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) for 15 min in vacuum. After release from vacuum, the tissues were further incubated in the fixative for 1 h at room temperature. Fixed floral tissues were embedded in Paraplast Plus (Sigma), and 8-mm sections were placed onto APTES-coated slides (Matsunami). Hybridization of dig-*SDG4* RNA probe was done at 45 °C for about 16 to 18 h.

Post-hybridization washings and signal detection were carried out according to the method of Tansengco et al. (2003). The hybridization sites were visualized using alkaline phosphatase conjugated anti-digoxigenin (Roche) and color development was monitored with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Slides were dried, mounted with glycerol and observed under a light microscope (Olympus BX50). Images were captured with a CCD camera (Olympus DP70) and processed using Adobe Photoshop (ver.7.0).

Western blot analysis

Histone-enriched protein extracts were prepared from floral tissues of Columbia and *sdg4* plants following the procedures by Tariq et al. (2003). The methylated histones were detected using the following antibodies purchased from Upstate Biotechnology: anti-dimethyl-H3K4, anti-trimethyl H3K4 anti-dimethyl-H3K9, anti-dimethyl H3K36 and anti-trimethyl H3K36 from Abcam. The relative histone H3 levels were normalized using an anti-histone H3 antibody (Upstate Biotechnology).

Immunofluorescence

Pollen grains were collected by vortexing open flowers in phosphate-buffered saline (PBS). After centrifugation, the supernatant containing floral tissues was discarded, and the pollen was fixed in 4% paraformaldehyde in PBS and treated with 0.5% Triton X-100 in PBS before incubation with the primary antibody (same antibodies as in western blot analysis). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Invitrogen). The pollen suspension was transferred to a microscope slide, counterstained with DAPI (1 μg/ml) in antifade and observed under a fluorescence microscope (Zeiss Axiophot). Images were captured using a CCD camera and enhanced to reduce background signals using Adobe Photoshop (ver.7.0).

Clearing intact siliques

A modified procedure by Howden et al. (1998) was followed for clearing intact siliques. Clearing was performed using lactoglycerol (2 water–2 glycerol–1 lactic acid) and staining with 0.1% aniline blue in lactoglycerol at 70 °C. After destaining in lactoglycerol at room temperature, the siliques were directly observed under a dissecting microscope (Leica MZFLIII).

In vitro pollen germination

Pollen germination assay was performed using the basic medium described by Fan et al. (2001). Pollen grains were transferred on the surface of the basic medium, and the Petri dishes were incubated in humid chambers at 22 °C overnight. The number of germinated pollens was counted and the pollen tube length was measured under a dissecting microscope.

Microarray analysis

Open flowers from wild-type and *sdg4* mutants were harvested and tested in an Agilent Arabidopsis 3 Oligo Microarray (Agilent Technologies). For each biological replicate, the materials from two to three plants were pooled to make a single sample for RNA purification. Two repetitive microarray experiments were done using the biologically independent RNA samples. Total RNA was isolated with Trizol reagent (Invitrogen) and were used for the preparation of Cy5- and Cy3-labeled cDNA probes. All microarray experiments, including the data analysis, were performed according to the manufacturer's manual (http://www.chem.agilent.com/Scripts/PCol.asp?lPage=494). Feature extraction and image analysis software (version 9.1.3.1; Agilent Technologies) was used to

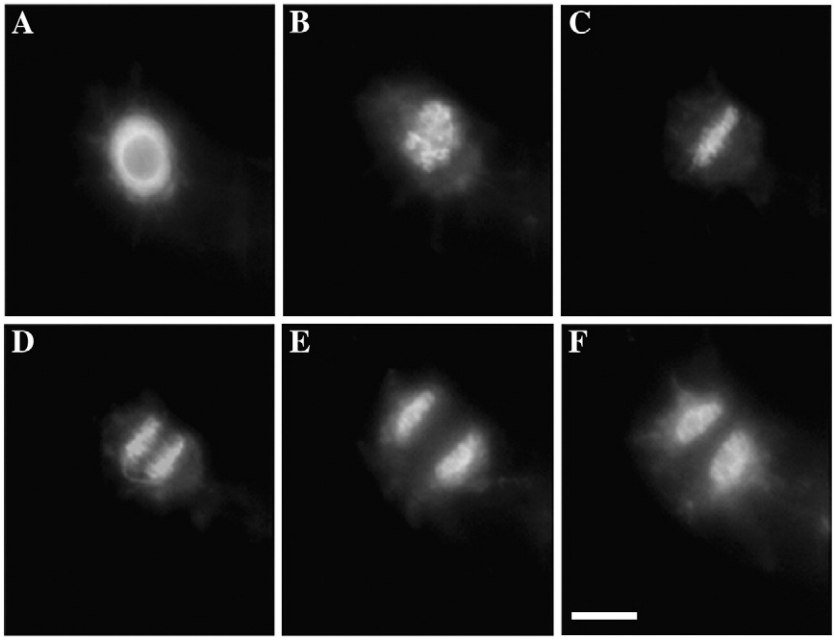


Fig. 2. SDG4 dynamics throughout the cell division of a transgenic tobacco BY-2 cell line. (A) Interphase; (B) prometaphase; (C) metaphase; (D) anaphase; (E) telophase; (F) cytokinesis. Scale bar=30 μm.

locate and delineate every spot in the array and to integrate each spot's intensity, filtering and normalization by the Lowess method. Gene clustering analysis was performed with Genespring 7.3.1 software (Agilent Technologies). On the basis of our empirical findings, expression of genes showing average signal intensity values of <1000 in channel was not always detected reproducibly by RNA gel blot analysis. For the identification of the genes upregulated in the mutant, the following 3 criteria were used in this study: (1) expression ratios (mutant/control) >2.0. (2) The genes show an average signal intensity >1000 for the mutant samples. (3) The data were not flagged with the flags that Agilent Technologies recommends (http://www.chem.agilent.com/Scripts/PHome.asp) except for glsBGPopnOL and rlsBGPopnOL. For the identification of the genes downregulated in the mutant, the following 3 criteria were used in this study: (1) expression ratios (mutant/control) <0.5. (2) The genes show an average signal value >1,000 for the wild-type control samples. (3) The data were not flagged with the flags that Agilent Technologies recommends (http://www.chem.agilent.com/Scripts/PHome.asp) except for glsBGPopnOL and rlsBGPopnOL.

Results and discussion

SDG4 encodes a nuclear protein with a SET domain and a PHD-finger motif

The amino acid sequence and domain organization of SDG4 is presented in Fig. 1A. Protein domain predictions using the Pfam database (http://www.sanger.ac.uk/Software/Pfam/index.shtml) indicated that amino acids 120–186 encode a PHD-finger, whereas a SET domain corresponds to amino acids 320–449. The presence of an NLS (amino acids 94–111) may suggest that SDG4 is targeted to the nucleus. The SET domain is associated with chromosomal proteins that regulate gene expression (Jenuwein et al., 1998). PHD-finger domains, on the other hand, have been proposed to be involved in complex formation or recognition of nuclear targets related to chromatin structure and regulation (Aasland et al., 1995). The protein domain organization displayed by SDG4 is characteristic of motifs commonly found in transcription factors.

SDG4 is classified under the ASH1 class of *Arabidopsis* SET domain proteins (Baumbusch et al., 2001). To show its homology to ASH1, amino acid sequence alignment of the SDG4 SET domain was performed against the SET domains of ASH1 from other organisms such as human (Nakamura et al., 2000), *Drosophila* (Tripoulas et al., 1996) and mouse (GenBank accession NP_619620) (Fig. 1B). The SET domain from human trithorax, TRX5 (GenBank accession CAB45386), was included in the alignment in order to demonstrate the presence of conserved motifs among SET domain-containing proteins. In addition to this, the ASH1 protein has the highest similarity to TRX among other SET domain proteins (Nakamura et al., 2000; Baumbusch et al., 2001). The GWG motif and cysteine-rich regions were shown to be conserved among the ASH1 and TRX proteins after amino acid sequence alignment by ClustalW (Fig. 1B). Accordingly, previously characterized plant PHD-finger proteins were also aligned with the PHD-finger of SDG4 (Fig. 1C). Among them are *Arabidopsis* HAT3.1 (Schindler et al., 1993), ZmHOX2a from *Zea mays* (Klinge et al., 1996) and MS1 (male sterility 1) from *Arabidopsis* (Wilson et al., 2001). The conserved C4HC3 motif for PHD-finger is highlighted in Fig. 1C. The results of the amino acid sequence alignment suggest that SDG4 possesses the conserved residues also found in other functional proteins and can serve as basis for predicting the SDG4 function.

SDG4 is a chromosome-associated protein

Using time-lapse video microscopy, the dynamic association of SDG4 expression with tobacco BY-2 chromosomes was

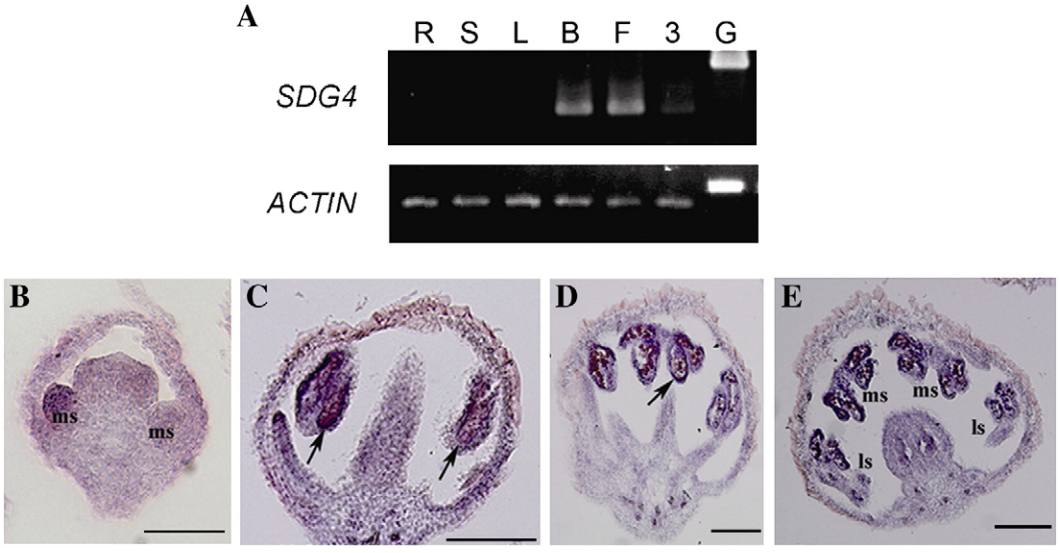


Fig. 3. *SDG4* expression analysis (A) Different tissues were examined for RT-PCR analysis of *SDG4* expression: root (R), stem (S), leaf (L), flower bud (B), mature flower (F), 3-day-old seedling (3), genomic DNA (G). RT-PCR using primers specific for *ACTIN* was done to serve as a control reaction. (B–E) In situ hybridization analysis of *SDG4* expression at different stages of flower development. *SDG4* transcripts accumulate in stamen primordia (B), anther locules (C) mature anther walls and pollen grains (D and E). (B) Stage 6 flower bud. (C) Stage 10 flower. Arrows show accumulation of *SDG4* transcripts in anther locules. (D) Stage 12 flower. Arrows indicate *SDG4* transcripts along anther walls. (E) Stage 12 flower. *SDG4* transcript level is relatively the same among lateral (ls) and medial (ms) stamens. Scale bars: A=50 μm, B–D=100 μm.

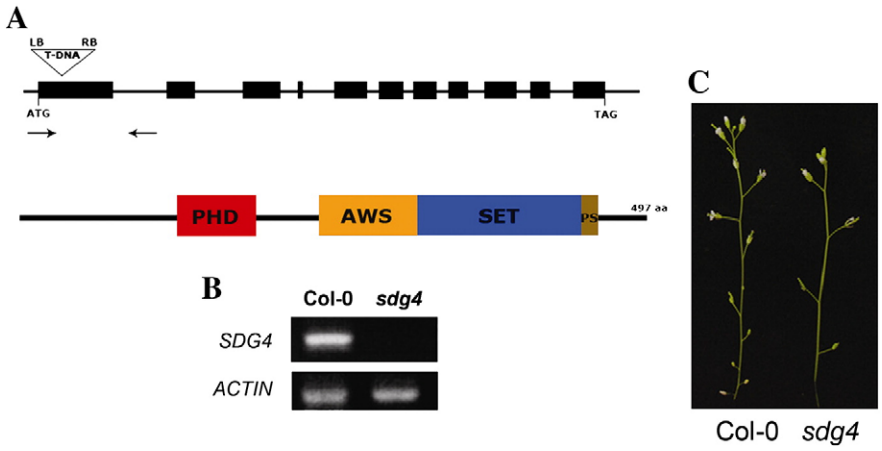


Fig. 4. Effect of *sdg4* mutation on flower development. (A) *SDG4* gene structure and position of T-DNA insertion. Black boxes: exons; black lines: introns; LB: T-DNA left border primer; RB: T-DNA right border primer. Arrows indicate the position of PCR primers used in verification of T-DNA insertion. Below: domain organization of the SDG4 protein. (B) RT-PCR analysis of *SDG4* expression in *sdg4* and wild-type flowers. (C) Comparison of bud production in wild-type and *sdg4* inflorescence.

established (Supplemental Data-Movie 1). Actively dividing tobacco BY-2 cells overexpressing SDG4 with a C-terminal GFP (GFP–SDG4) were monitored, and the GFP signal was shown to be mainly localized in the chromatin during interphase and throughout mitosis. Fig. 2 illustrates the localization pattern of SDG4 at different stages of cell division. At interphase, a strong GFP fluorescence was detected in the entire nucleus, except the nucleolar area (Fig. 2A). As the chromatin started to condense, the GFP signal was shown to be associated with the condensing chromatin starting prometaphase (Fig. 2B). Strong GFP signals on highly compacted chromosomes were observed during metaphase (Fig. 2C). As the cells reach anaphase, the separation of GFP signals on each of the chromosome sets was evident (Fig. 2D); and immediately after that, dynamics of the GFP signal is consistent with the complete separation and movement of the chromatids to opposite sides of the cells at telophase (Fig. 2E). GFP–SDG4 expression of the chromatin was evident until the cells reach cytokinesis (Fig. 2F). Tobacco BY-2 cells overexpressing SDG4 with an N-terminal GFP (SDG4–GFP) showed exactly the same localization pattern as that of GFP–SDG4 (data not shown). The correlation of SDG4 expression to chromosomes during cell division and the presence of both the SET and PHD-finger domains in SDG4 provide considerable information for the possible involvement of SDG4 in chromatin remodeling.

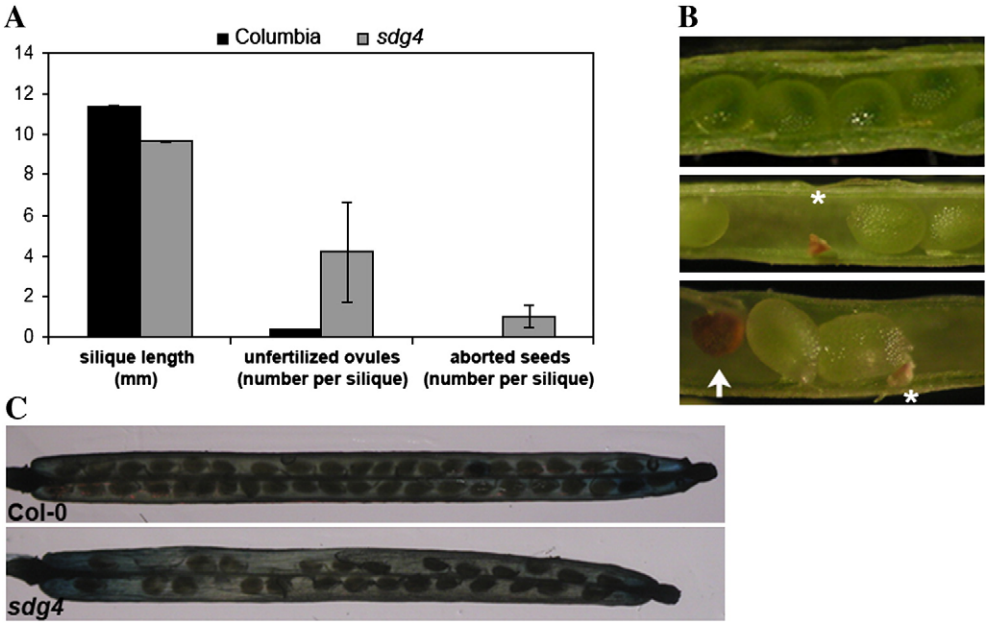


Fig. 5. Unfertilized ovules and aborted seeds were frequently observed in *sdg4* siliques. (A) The silique length was measured and the number of unfertilized ovules and aborted seeds were counted in wild type and *sdg4*. (B) Wild-type silique containing healthy seeds (top panel) and *sdg4* siliques (middle and bottom panels) having unfertilized ovules (arrows) and aborted seeds (asterisks) (C) Cleared siliques showing seed distribution in both wild type and *sdg4*.

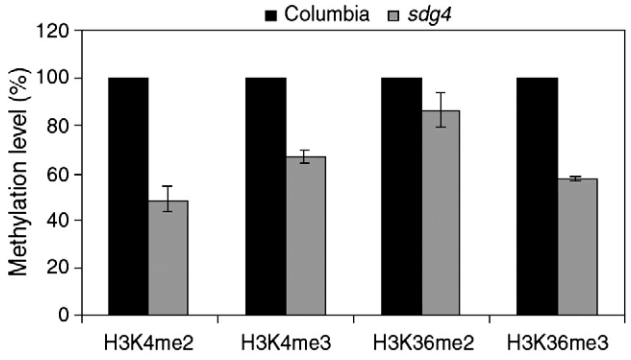


Fig. 6. Histone methylation levels were reduced in *sdg4* mutants. Western blot analysis of methylated H3K4 and H3K36 in the inflorescence of wild-type and *sdg4* mutant. Values were normalized using an anti-histone H3 antibody. $n=3$.

SDG4 is highly expressed in the pollen

The results of RT-PCR analysis using *SDG4* and *ACTIN* primers are presented in Fig. 3A. *SDG4* was shown to be expressed at high levels in mature flowers and flower buds at relatively equal amounts, indicating that *SDG4* is likely to be expressed throughout the different stages of *Arabidopsis* flower development. Detectable expression was also observed in 3-day-old seedlings. Furthermore, increasing the number of PCR cycles to 40 detected *SDG4* transcripts from the other organs analyzed, such as roots, stems and leaves (data not shown).

To facilitate the observation of tissue-specific expression of *SDG4* in *Arabidopsis*, *in situ* hybridization to floral tissues was carried out using an *SDG4* antisense probe. *SDG4* expression was monitored at different stages of flower development. *SDG4* transcripts were below detection levels in early floral primordia (data not shown). Expression of *SDG4* in the stamen primordia was first detected among stage 6 flowers (Fig. 3B). Accumulation of *SDG4* transcripts was observed within anther locules of flowers at stage 10 (Fig. 3C), and along anther walls and pollen grains in a stage 12 flower bud (Figs. 3D and E). The level of *SDG4* expression was found to be almost the same in the lateral and medial stamens (Fig. 3E). Slightly

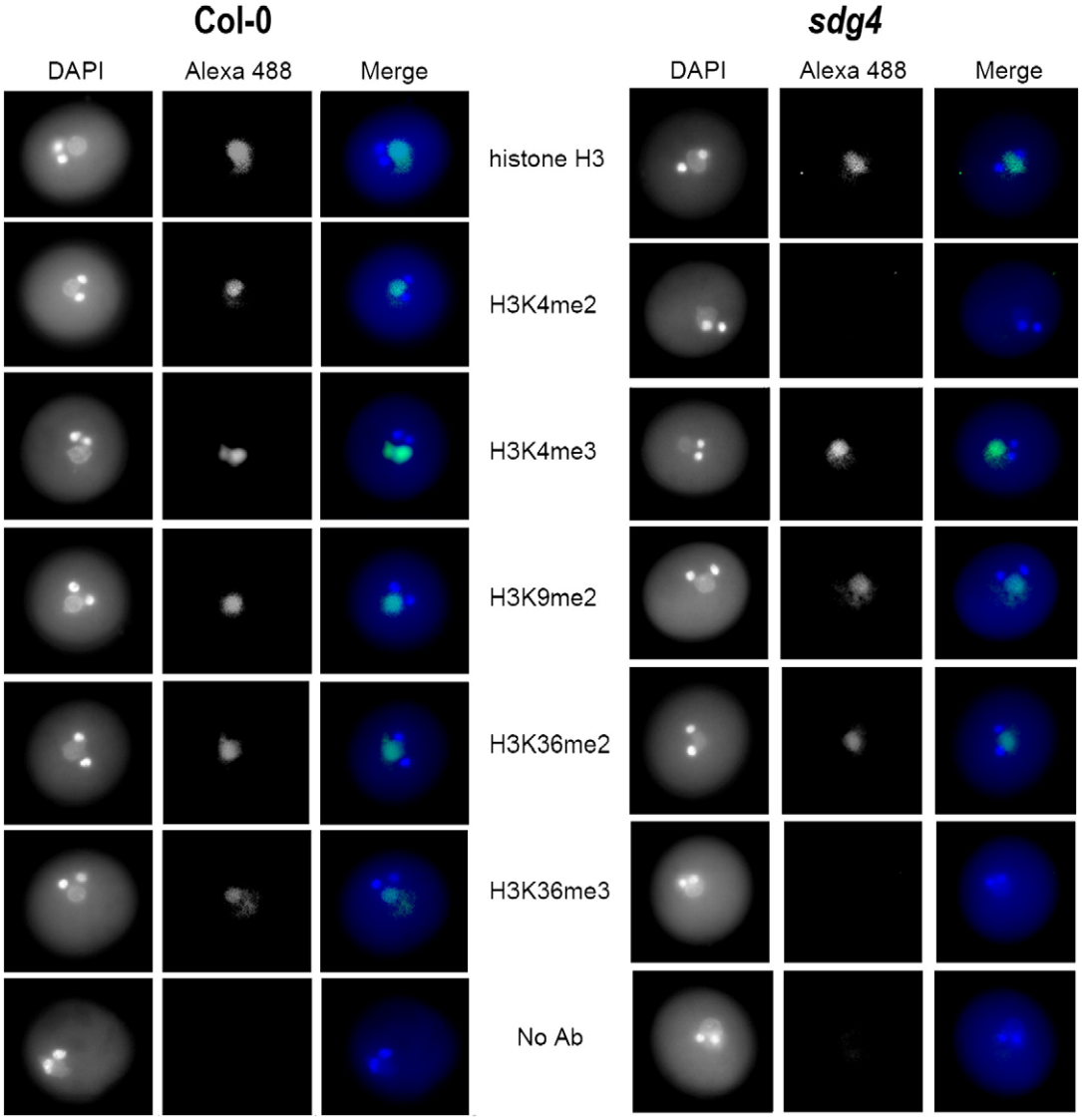
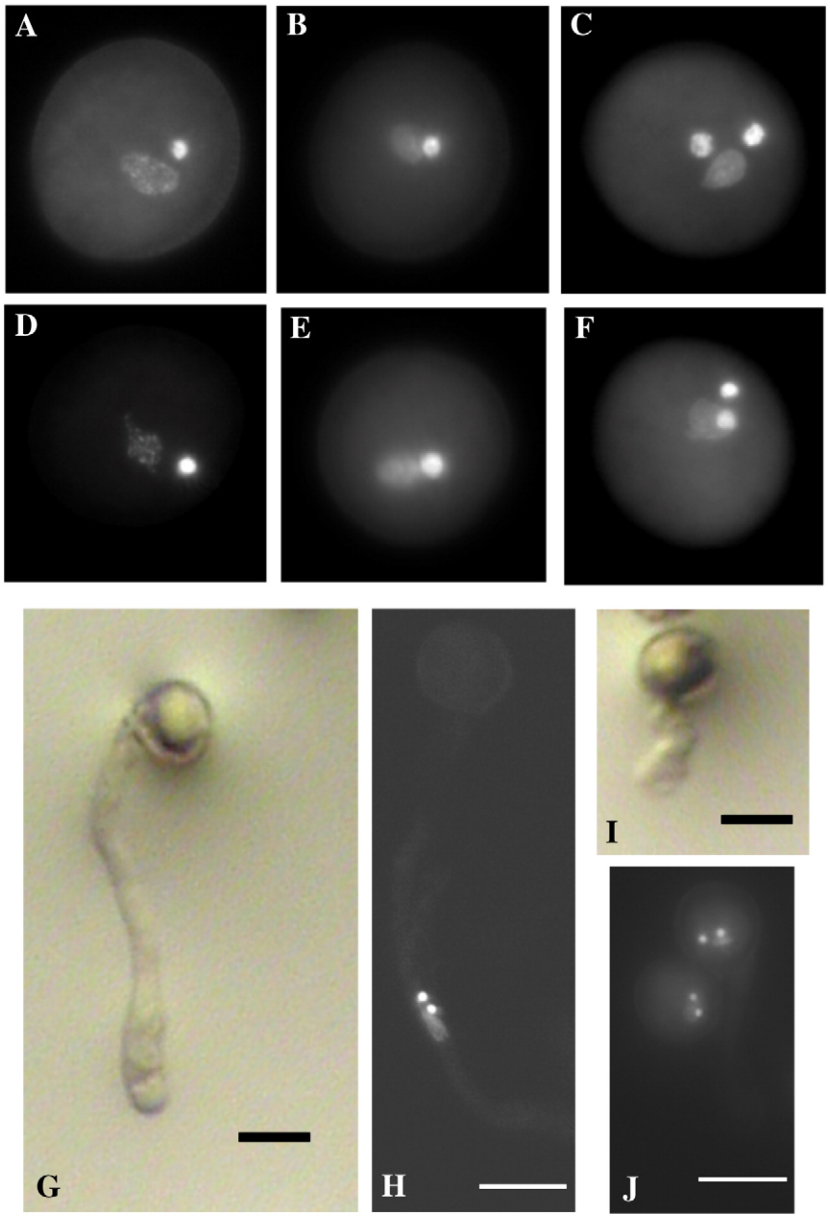


Fig. 7. Immunofluorescence staining using specific antibodies against unmodified and methylated histone H3 in wild-type and *sdg4* pollen.


A
B
C
D
E
F
G
H
I
J
K
Pollen tube length (μm)
400
300
200
100
0
Col-0 (+/+)
heterozygous (+/-)
homozygous (-/-)

detectable levels of *SDG4* transcripts were also observed in the stamen filaments (Figs. 3D and E), indicating that *SDG4* is expressed in the entire stamen structure.

Arabidopsis microarray data on the tissue-specific expression of *SDG4* was obtained from GENEVESTIGATOR (https://www.genevestigator.ethz.ch/at/index.php) and indicated that *SDG4* is highly expressed in the pollen (Zimmermann et al., 2004). Furthermore, the expressions of two other *Arabidopsis* ASH1-related genes and three ASH1 homologs were also obtained from GENEVESTIGATOR. Comparing the signal intensities for expression of these six genes in pollen, it was shown that the high expression in pollen is exclusive for *SDG4* (Fig. S1). The average signal intensity value for *SDG4* was found to be 34,195 whereas less than 1,000 for the other five genes. This excludes the possibility of redundancy in SDG4 function as a major ASH1 protein that acts as a histone methyltransferase in *Arabidopsis* pollen.

A mutation in the SDG4 gene affects the fertility of Arabidopsis flowers

In order to identify the possible function of *SDG4* in *Arabidopsis* development, a homozygous mutant line harboring a T-DNA insertion was characterized (Fig. 4A). The predicted site of T-DNA insertion for SALK 128444 on the first exon of the *SDG4* gene was confirmed by PCR and sequence analysis (data not shown). The T-DNA insertion greatly reduced the level of *SDG4* transcripts as analyzed by RT-PCR (Fig. 4B). However, longer unexpected transcripts could be detected in open flowers of *sdg4* mutants by Northern hybridization using a DNA probe (Fig. S2). Although the transcript level increases three to five times, these transcripts might have been transcribed together with the T-DNA insertion by the SDG4 promoter and spliced from the long premature RNA. These transcripts cannot produce the functional SDG4 protein since they lack an inframe initiation codon. Upon inspection of the vegetative organs in these *sdg4* homozygous plants, no apparent morphological abnormalities were observed, except that the rate of bud production was lower than the wild type (Fig. 4C).

The presence of a T-DNA insertion in the first exon of the *SDG4* gene is expected to disrupt the expression of the functional domains in the SDG4 protein, such as the PHD and SET domains (Fig. 4A). Previous reports on the roles played by SET-containing (Goodrich et al., 1997; Grossniklaus et al., 1998; Alvarez-Vanegas et al., 2003) and/or PHD-containing (Wilson et al., 2001; Reddy et al., 2003; Yang et al., 2003) proteins include the regulation of flower development. Recently identified PHD-containing *Arabidopsis* proteins, such as MS1 (Wilson et al., 2001; Ito and Shinozaki, 2002) and MMD1/DUET (Reddy et al., 2003; Yang et al., 2003), were shown to function in anther and pollen development. Thus, male meiosis and pollen development were analyzed in *sdg4* flowers. However, pollen from flowers of the homozygous plants did not show any significant morphological difference when compared to the wild-type pollen. Mature pollen from both wild-type and *sdg4* mutants contain two sperm nuclei and one vegetative nucleus as visualized by DAPI staining (Figs. 8A–F). Likewise, meiotic chromosomes from the *sdg4* homozygous line appeared morphologically the same as that of the wild type (Fig. S3).

Though pollen development was normal in *sdg4* mutants, the fact that significant amount of *SDG4* transcripts were found in the pollen indicates that SDG4 function is concerned with processes taking place in the pollen. After germination, the pollen grows a tube that is used to deliver the sperm cells in order to reach the female gametophyte for fertilization. Thus, we tried to investigate the role of *SDG4* in the reproductive development in *Arabidopsis*. A general observation on the silique length among *sdg4* mutants gave us an impression that mutant siliques appeared slightly shorter than that of the wild type. Silique length measurements were done on the first five siliques found in each stem, and our analysis indicated that *sdg4* siliques are about 2 mm shorter in average than that of wild type (Fig. 5A). Furthermore, fresh siliques were dissected for analysis of ovule development. The *sdg4* siliques were found to contain unfertilized ovules and aborted seeds (Figs. 5A–B). The number of unfertilized ovules and aborted seeds was counted in both wild-type and *sdg4* siliques and was found to vary widely among *sdg4* siliques even within the same plant (Fig. S4). However, wild-type siliques contained very few unfertilized ovules and almost no aborted seeds (Fig. 5A). Clearing of intact siliques showed clearly that the number of healthy seeds contained in *sdg4* siliques is lower than in the wild type (Fig. 5C).

The increased number of unfertilized ovules in *sdg4* compared to wild type indicates that fertility is affected by *sdg4* knockout. Furthermore, the increased variations in the number of unfertilized ovules indicate that this phenotype is most likely due to epigenetic changes in gene expression. Several genes expressed in the pollen play vital roles in assuring that fertilization can take place successfully. Three independent studies on the global gene expression in the *Arabidopsis* pollen have shown that the pollen contain genes that mainly function in signaling, cell wall metabolism and cytoskeletal dynamics (Honys and Twell, 2003; Becker et al., 2003; Lee and Lee, 2003). These gene families are expected to be involved in pollen germination, pollen tube growth and guidance. Alteration in the expression level of these genes can drastically influence the success or failure of fertilization. SDG4, being a SET domain transcription factor has a potential for regulating gene expression through chromatin remodeling, specifically by methylation of specific lysine residues.

Fig. 8. Pollen development in *sdg4* mutants appeared normal and comparable with that in Columbia. DAPI staining to illustrate pollen development in Columbia (A–C) and *sdg4* (D–F). A and D are at early bicellular; B and E are at mid bicellular; C and F are at tricellular/mature stage. *In vitro* germinated pollen grains from Columbia (G) and *sdg4* (I) showing difference in pollen tube lengths. DAPI staining to track the movement of the sperm and vegetative nuclei in germinated wild-type (H) and *sdg4* (J) pollen (scale bars = 20 μm). (K) Pollen tube lengths for Columbia, *sdg4* heterozygous and *sdg4* homozygous mutants were measured 24 h after incubation in pollen germination medium. The mean value for each population is indicated by a horizontal line.

SDG4 modulates the level of methylated histone H3 in the vegetative nucleus of Arabidopsis pollen

The SET domain of SDG4 is most similar in homology to *Drosophila* ASH1 (Springer et al., 2003; Baumbusch et al., 2001). The specific enzymatic activity established for *Drosophila* ASH1 includes methylation of H3K4, H3K9 and H4K20 (Beisel et al., 2002; Byrd and Shearn, 2003), and recently H3K36 (Tanaka et al., 2007). Histone methyltransferase assays were performed for recombinant SDG4 proteins, but *in vitro* activities were not detected (data not shown). This indicates that like some SET domain methyltransferase proteins, SDG4 requires association with other proteins in a complex in order to carry out its enzymatic activities (Jenuwein, 2006). Thus, the level of methylated histones in the wild-type and *sdg4* mutant flowers were analyzed by immunoblotting with specific anti-methyl antibodies. In the *sdg4* mutant line, a significant reduction in the level of dimethylated H3K4, trimethylated H3K4 and trimethylated H3K36 was evident (Fig. 6; Fig. S6). On the other hand, the dimethylated H3K36 level was only slightly reduced in the *sdg4* mutant (Fig. 6; Fig. S6). This ability of SDG4 to methylate the lysine 4 residue of histone H3 is in agreement with the reported enzymatic activity of ASH1 proteins in both human and *Drosophila* (Beisel et al., 2002; Byrd and Shearn, 2003).

ASH1 proteins are chromatin remodelers specifically acting on H3K9 and H3K4. In *Drosophila*, transcriptional activation by ASH1 corresponds to methylation of lysines 9 and 4 in H3 and lysine 20 in H4 at the promoter of ASH1 target genes (Beisel et al., 2002). Furthermore, it was demonstrated that ASH1 is responsible for all *in vivo* histone H3K4 methylation in *Drosophila*; and that the SET domain alone is sufficient for its histone methyltransferase activity (Byrd and Shearn, 2003). A recent report by Tanaka et al. (2007) provides evidence that histone H3 K36 is specifically methylated by mammalian and *Drosophila* ASH1. This histone H3 K36-specific methyltransferase activity was demonstrated in *Arabidopsis* by the ASH1 homolog, SDG8 (Zhao et al., 2005). SDG8 was shown to have a specific activity to methylate K36 of histone H3 in order to activate the expression of *FLOWERING LOCUS C* (*FLC*). Considering these characteristic methylation activities of different ASH1 proteins so far characterized, we can rationalize that SDG4 transcriptional activation involves methylation of histone H3 K4 and K36.

The significantly high expression level of SDG4 in pollen prompted us to analyze histone methylation levels in pollen grains by immunofluorescence staining using antibodies against specific methylation marks. The antibodies used for pollen immunostaining detected the tissue-specific histone protein localized exclusively in the vegetative nucleus. All methylation marks were clearly immunolocalized in the vegetative nucleus of wild-type pollen (Fig. 7). In *sdg4* pollen, the dimethyl H3K4 and trimethyl H3K36 signals in the vegetative nucleus were greatly reduced whereas the other methylation marks were not

Table 1
Genes downregulated in *sdg4* mutant flowers

Functional category	No. of genes	Description	MIPS number
Cell wall metabolism	4	Pectinesterase family protein, xyloglucan: xyloglucosyl transferase (putative), glycosyl transferase family 8 protein	At2g45220, At4g14130, At3g25050, At4g02130
Transcription	2	AP2 domain-containing transcription factor family protein, myb family transcription factor	At5g61590, At3g46640
Transferase activity	1	Transferase family protein similar to hypersensitivity-related gene product HSR201	At5g41040
Response to abiotic or biotic stimulus	4	Senescence-associated protein (SEN1), encodes a member of glycosyl hydrolase family 36. Raffinose synthase family protein/seed imbibition protein, glycosyl hydrolase family 1 protein, pseudo-response regulator 5 (APRR5)	At4g35770, At5g20250, At3g60140, At5g24470
Transporter activity	1	Organic cation transporter-related low similarity to organic cation/carnitine transporter 2	At1g16390
Receptor binding or activity	4	Caleosin-related family protein similar to Ca^{2+}-binding EF hand protein, chlorophyll A–B binding protein 2, glycine-rich protein (GRP17) olesin, pollen recognition, serine protease inhibitor	At1g70680, At1g29930, At5g07530, At4g01575
Response to stress	1	26.5-kDa class P-related heat shock protein	At4g21870
Transport	1	Protease inhibitor/seed storage/lipid transfer protein (LTP) family	At1g66850
Other enzyme activity	4	Peroxidase 21 (PER21), cysteine proteinase, F-box family protein (FKF1)/adagio 3 (ADO3) E3 ubiquitin ligase SCF complex F-box subunit	At2g37130, At1g06260, At5g50260, At1g68050
Developmental processes	1	Selenium-binding protein, putative	At4g14040
Unknown protein	14	Unknown	At3g01345, At1g53480, At2g20670, CHR3:013881301-013881242, At1g04540, At2g33830, At5g09530, At5g45880, At2g40330, At1g80440, At4g37540, At2g05540, At3g01290, At4g28040

Table 2
Genes upregulated in *sdg4* mutant flowers

Functional category	No. of genes	Description	MIPS number
Response to stress	12	17.6-kDa class I heat shock protein (HSP17.6A-CI), heat shock protein 101 (HSP101), heat shock protein 70, putative, DNAJ heat shock family protein, heat shock protein 81-1 (HSP81-1), 17.4-kDa class III heat shock protein (HSP17.4-CIII), stress-responsive protein, putative	At1g59860, At1g07400, At1g74310, At2g29500, At3g12580, At2g20560, At5g52640, At1g54050, At2g32120, At3g14200, At3g16050, At4g12400
Transcription	16	Heat shock transcription factor family protein, ethylene-responsive transcriptional coactivator (putative), AP2 domain-containing transcription factor (putative), DRE-binding protein (putative), WRKY family transcription factor, transcription regulator, homeobox-leucine zipper protein 12 (HB-12), zinc finger (C3HC4-type RING finger) family protein, SET domain-containing protein, elongation factor 1B alpha-subunit 1	At2g26150, At3g24500, At3g24500, At1g19210, At1g12610, At1g80840, At4g23810, At5g21960, At1g26945, At3g51910, At3g61890, At1g74930, At4g34410, At1g14200, At4g30860, At5g05410, At5g12110
Signal transduction	5	Heptahelical transmembrane protein homologous to human adiponectin receptors and progestin receptors, seven transmembrane MLO family protein, WD-40 repeat family protein, calmodulin-related protein (putative)	At4g30850, At2g39200, At1g78070, At5g42380, At3g01830
Transferase activity	5	Transferase family protein, glutaredoxin family protein, glutathione S-transferase, glucosyltransferase-related	At2g19070, At5g38130, At1g28480, At2g29450, At4g16590
Response to abiotic or biotic stimulus	6	Ethylene-responsive element-binding protein (putative), allene oxide cyclase (putative), disease resistance protein (putative), chlorophyll A–B binding family protein, immediate–early fungal elicitor family protein, auxin-responsive GH3 family protein	At2g44840, At3g25780, At1g66090, At3g22840, At3g02840, At2g46370
Kinase activity	1	Lectin protein kinase, putative	At1g70130
Developmental processes	3	Late embryogenesis abundant protein (putative), nodulin MtN3 family protein, 2-oxophytodienoate reductase (OPR3)/delayed dehiscence1 (DDE1)	At1g52690, At4g25010, At2g06050
Transport	2	Lipid transfer protein (putative), ABC transporter family protein	At3g51590, At1g15520
Cell wall metabolism	4	Pectinesterase family protein, invertase/pectin methylesterase inhibitor family protein, xyloglucan: xyloglucosyl transferase (putative)	At5g07420, At3g17130, At1g70720, At4g30280
Electron transport or energy pathways	8	Cytochrome P450 family protein, allene oxide synthase (AOS)/hydroperoxide dehydrase/cytochrome P450 74A, cytochrome P450 family protein, NADP-dependent oxidoreductase (putative)	At3g48520, At5g63450, At5g42650, At3g25180, At5g52400, At1g69500, At2g27690, At5g16960
Nucleic acid binding	1	Zinc finger (C2H2 type) family protein	At5g59820
Other cellular processes	1	Circadian clock coupling factor (putative)	At3g63060
Other biological processes	16	Caffeoyl-CoA 3-*O*-methyltransferase (putative), GDSL-motif lipase/hydrolase family protein, myrcene/ocimene synthase (TPS10), rapid alkalinization factor (RALF) family protein, phosphatidic acid phosphatase-related, ankyrin repeat family protein, 1-aminocyclopropane-1-carboxylate synthase 6, 4-coumarate-CoA ligase family protein/4-coumaroyl-CoA synthase family protein, 9-*cis*-epoxycarotenoid dioxygenase (putative), gibberellin 2-oxidase, putative/GA2-oxidase (putative), transformer serine/arginine-rich ribonucleoprotein (putative), armadillo/beta-catenin repeat family protein, acyl-activating enzyme 11 (AAE11), ChaC-like family protein, peptidyl-prolyl *cis*-trans isomerase (putative), lipoxygenase (putative)	At1g67990, At5g45950, At2g24210, At4g14020, At3g54020, At2g24600, At4g11280, At1g20510, At3g14440, At1g02400, At1g07350, At3g09350, At1g66120, At5g26220, At5g48570, At1g72520
Unknown	29	Unknown	At2g46240, At2g22880, At4g27654, At5g62520, At4g27657, At1g56660, At1g66080, At3g07090, At1g20310, At1g19180, At4g24380, At1g17380, At2g34600, At3g06890, BX824487, At2g17660, At5g02580, At2g19310, At1g52565, At4g16515, At1g30135, At4g01360, At5g50335, At5g19230, At5g10695, At5g50360, At4g29780, At5g13220, At5g03210.1

affected. This methylation pattern is consistent with the immunoblotting results pointing to a transcriptional activation mode of histone modification by SDG4.

Histone proteins are generally categorized into three: replication dependent, replication independent and tissue specific (Schumperli, 1986). In lily, tissue-specific histone variants such as gH2A, gH2B and gH3 were reported to be specifically localized in the generative cells of pollen grains (Ueda and Tanaka, 1995; Xu et al., 1999; Okada et al., 2006a). Moreover, the male gamete-specific histone gcH3 in lily has been shown to possess a promoter that is activated independent of vegetative cell transcriptional and translational machineries (Okada et al., 2005a). More recently, a histone variant H3.3 was shown to be particularly expressed in the vegetative nucleus of lily pollen (Sano and Tanaka, 2005, 2007). It was also demonstrated that this histone H3.3 variant was preferentially incorporated into chromatin, where it is expected to control chromatin condensation and upregulate genes that are necessary for pollen germination and tube growth (Sano and Tanaka, 2005, 2007). Meanwhile, 15 histone H3 genes have been identified in *Arabidopsis* by a bioinformatic approach, including one that is a male-gamete-specific variant (Okada et al., 2005b). Ingouff et al. (2007) has studied the dynamics of this male-gamete-specific H3.3 through live imaging of the fertilization process in *Arabidopsis thaliana*. The histone H3 protein we detected here is specifically localized in the vegetative nucleus and is distinct from the male-gamete-specific H3.3 previously described by Ingouff et al. (2007).

The nucleus of the vegetative cell is said to be more transcriptionally active than that of the generative cell since numerous pollen-specific genes that function in pollen germination and tube growth are expressed in the vegetative cell (Honys and Twell, 2003). In contrast, genes that function in protein destination and signal transduction were found to be abundant in the generative cell of lily pollen (Okada et al., 2006b, 2007). Since we detected histone methylation in the vegetative nuclei, we then tried to analyze pollen germination as well as pollen tube growth in *sdg4* mutants. The rate of pollen germination did not differ between wild type and *sdg4* (data not shown). However, the growth of pollen tube was affected in *sdg4,* in which most of the *sdg4* pollen tubes did not reach the normal length as in wild type even after 24 h of incubation (Fig. 8K). Wild-type pollen tubes have an average length of about 150.0 μm, whereas homozygous *sdg4* pollen tubes reached about 78.8 μm in length. The distribution plot in Fig. 8K shows that the measured pollen tube lengths for heterozygous mutants cover the range of tube length values for both wild-type and homozygous *sdg4* pollen, with the longer pollen tubes lengths comparable to that of wild-type (average length of 149.1 μm) and shorter pollen tubes similar to that of *sdg4* homozygous mutants (average length of 54.6 μm).

DAPI staining allowed us to visualize the vegetative and sperm nuclei during pollen development and to track the nuclei along the pollen tube after germination (Figs. 8A–J). In *sdg4* mutants, the sperm and vegetative nuclei remained in the pollen grain (Fig. 8J), whereas all these nuclei were located near the tip of wild-type pollen tube as if ready to be delivered out into the ovules (Fig. 8H). The defect in pollen tube growth among *sdg4* mutants resulted to failure of the sperm cells to reach the ovules for fertilization; thus, numerous ovules remained unfertilized. Based on these findings, we conclude that SDG4 is capable of regulating the pollen tube growth in *Arabidopsis* by altering the expression of pollen-specific genes via histone methylation.

To validate such conclusions, we performed microarray experiments to analyze gene expression in *sdg4* mutant flowers. The microarray data we obtained were assigned the GEO accession number GSE9543. Table 1 presents the genes downregulated by *SDG4* mutation. In the list, several genes involved in cell wall metabolism, stress response and transport, as indicated by database annotations, were found to have decreased expression in *sdg4* mutants. Genes involved in cell wall synthesis are also implicated in pollen tube growth, such as pectinesterases and glucosyl transferases. For the upregulated genes, those involved in transcription, stress response and signal transduction showed increased expression in *sdg4* mutant flowers (Table 2). We also found SDG4 as an upregulated gene with about three times increased transcription as compared to that in the wild type. This is consistent with our observation that the longer transcripts of *SDG4* were detected by Northern hybridization. In both cases, genes with increased or decreased expression levels were detected which are assumed to be associated with pollen development, especially affecting pollen tube growth. Thus, from these findings, it can be deduced that the downregulation of genes involved in pollen tube growth was due to the absence of SDG4.

Histone modification is a major factor that controls the chromatin state in many organisms, which subsequently affects gene expression and development. The current paper presents novel findings that *Arabidopsis* ASH1-related protein, SDG4, is involved in histone modifications of H3K4me2, H3K4me3 and H3K36me3, which are associated with the transcriptionally active chromatin state. A significant reduction in the level of methylated histone H3 K4 and K36 may have contributed to the observed defects in pollen tube growth, which subsequently affected fertility in *sdg4* mutants. The expression of genes that function in the growth of pollen tubes, most likely those involved in cell wall metabolism and signal transduction, were suppressed mainly by the significant reduction or loss of histone H3 K4 and K36 levels in the *sdg4* pollen. As a result, the sperm nuclei failed to reach the embryo sac, thus producing unfertilized ovules. The histone modification alteration shown in *sdg4* mutants is consistent with those for *Drosophila* and mammalian ASH1 proteins, thereby providing a link between animal and plant systems in terms of epigenetic development. This work paves the way for further investigations on the mechanism by which HMTase regulates expression of genes that are key players in plant reproduction.

Acknowledgments

We are grateful to Dr. Makoto Hayashi (Munich University) for the use of microscope and microtome in his laboratory; Dr. Tsuyoshi Nakagawa (Shimane University) for providing the pGWB-GFP vectors; Prof. Masao Tasaka and Dr. Mitsuhiro

Aida for their technical advice; Dr. Nirianne Palacpac for the helpful suggestions; Junko Ishida, Taeko Morosawa, Jutamart Khunsook, Reiko Sato and Reiko Isobe for the technical assistance extended. This work was supported by the Special Coordinations Funds of the Ministry of Education, Culture, Sports, Science and Technology and Japan Science and Technology Agency.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.12.016.

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